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(54) Title: METHOD OF MONITORING ENDOSPORE-FORMING BACTERIA

(57) Abstract

Endospore-forming bacteria present in a sample are detected or identified by adding to the sample primers which are specific for conserved regions in a coding region in the genome of the bacteria, and carrying out a DNA amplification procedure. In the detection method, any of the conserved regions present in the sample may be amplified, followed by analysing for the presence of the conserved regions. In the identification method, the conserved regions of the coding region are amplified, followed by analysing the amplified DNA obtained, the primers being such that they simultaneously amplify a variable coding sequence of DNA intermediate the conserved regions of the coding region, the length of the DNA fragment obtained from the amplification step being characteristic of a particular endospore-ming bacterium.

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Method of Monitoring Endospore-Forming Bacteria

The present invention relates to a method of monitoring endospore-forming bacteria.

Endospore-forming bacteria are important contaminants of food. Some of these bacteria are highly virulent pathogens, such as *Clostridium botulinum* and *Clostridium perfringens*. Bacterial spores are difficult to eliminate because they are resistant to heating and many other sterilising treatments. They can be introduced at almost any stage during food manufacture in raw materials or as a result of careless handling, lack of hygiene or the like. Constant monitoring of raw materials, food production plants, factories or the like is therefore necessary to guard against gross contamination of food by these pathogens.

The *spoOA* gene (or related homologs) is believed to occur in many Gram-positive endospore-forming bacteria such as *Bacillus* and *Clostridium* and to be absent from all non endospore-forming bacteria. The SpoOA protein product of the gene is a member of the response regulator super-family of "two-component" signal transduction proteins. The SpoOA protein is an ambiactive transcription factor which is activated by phosphorylation to stimulate or repress expression of specific sets of target genes. In particular, the phosphorylated form of the SpoOA protein directly controls initiation of endospore formation and also influences, typically via an indirect mechanism, other stationary phase phenomena in endospore-forming organisms.

The SpoOA protein has highly conserved regions within its carboxy and amino terminal domains, which domains are connected by a linker region, which region varies in sequence and length depending on the organism.

Likewise, another gene, *degU* (or a related homolog) concerned with regulation of extracellular enzyme production in endospore-forming bacteria has also been found to have a conserved two-domain structure connected by a variable linker region.

It has also been found that gene organisation in endospore-forming bacteria is substantially conserved. The highly conserved genes are connected by intergenic linker (non coding) regions of variable length, depending on the organism.

We have found that these conserved regions provide an effective marker for monitoring endospore-forming bacteria and that the variable linker regions separating the conserved regions provides highly specific markers for identifying individual species of endospore-forming bacteria.

Accordingly, there is provided by one aspect of the present invention, a method of monitoring endospore-forming bacteria present in a sample, which method comprises the steps of adding to the sample primers which are specific for conserved regions in a coding region in the genome of said bacteria, which coding region comprises two domains with a linker region connecting the two domains and which coding region is characteristic of an endospore-forming bacterium, carrying out a DNA amplification procedure so as to amplify conserved regions of the coding region present in said sample; and analysing for the conserved regions. Thus, advantageously, it is possible by using DNA amplification technology to monitor the presence of endospore-forming bacteria which might be present in a given sample. The presence of DNA corresponding to the conserved regions is indicative of the presence of endospore-forming bacteria.

In one embodiment of the invention, the conserved regions comprise segments of the amino and carboxy terminal domains of a single gene, such as *spoOA* or *degU*, the conserved regions being linked by the variable linker region. In an alternative embodiment of the invention, the conserved regions of the coding region comprise segments of separate adjacent genes such as *spoIVB* and *spoOA* whose disposition in the genome of the endospore-forming bacteria is highly conserved, and which genes are connected by a variable intergenic linker region.

According to a second aspect of the present invention, there is provided a method of identifying endospore-forming bacteria, which method comprises the steps of adding primers to

a sample containing endospore-forming bacteria, said primers being specific for the conserved regions of a coding region in the genome of the bacteria, which coding region is characteristic of an endospore-forming bacterium, carrying out a DNA amplification step so as to amplify conserved regions of the coding region and analysing the amplified DNA obtained, wherein said primers are such that they simultaneously amplify a DNA linker region of variable length connecting the conserved regions of the coding region, the length of the DNA fragment obtained from said amplification step being characteristic of a particular endospore-forming bacterium.

Thus, advantageously, endospore-forming bacteria which may be present in a given sample may be identified, based on the length of the variable DNA fragment connecting the conserved regions of the coding region, the linker region being amplified by the primers in addition to the conserved regions.

In one embodiment, the conserved regions of the coding region comprise segments of the amino and carboxy terminal domains of a single gene, such as *spoOA* or *degU*, the conserved regions being separated by the variable linker region. Alternatively, the conserved regions comprise segments of separate adjacent genes whose disposition in the genome of the endospore-forming bacteria is highly conserved and which genes and their disposition in the genome are characteristic of a particular endospore-forming bacterium, the genes being connected by an intergenic non-coding region.

Preferably, the amplification step comprises the polymerase chain reaction, isothermal amplification or the like. Thus, very small numbers of organisms present in the sample may be identified. The sample may be a clinical specimen, foodstuff or the like.

The invention may be more clearly understood with reference to the following examples.

Example 1

A pair of primers TIAARCCITTYGA and TAICCYTTIATRTGIGCIGGIACICC complementary to residues 107-111 (LKPFD) and 168-174 (PAHIKGY - reverse) of the deduced amino acid sequence of *spoOA* from *B. subtilis* were used to amplify DNA segments from several bacilli and clostridia using a Perkin Elmer GeneAmp™ PCR System 2400. Each reaction tube contained 100 pmole of each primer, 0.5μg of target DNA, all four dNTPs at 200μM each, 50mM KCl, 2mM $MgCl_2$, 10mM Tris/HCl, 0.001% gelatin (Sigma), pH 8.0, and 2.5U of AmpliTaq DNA polymerase (Perkin Elmer Cetus). After denaturation for 5 minutes at 94°C samples were amplified for 35 cycles comprising a 40 second denaturation period at 94°C, a 40 second annealing period at 40°C and a 40 second extension period at 72°C. Amplified products were analysed by electrophoresis through a 1.5% agarose gel. The results obtained are given in Table 1.

Table 1

Organism	Approximate Size of Amplified DNA Fragment
Bacillus subtilis	205
Bacillus cereus	195
Bacillus stearothermophilus	185
Bacillus megaterium	190
Bacillus brevis	180
Clostridium beijerinckii	215
Clostridium butyricum	230
Clostridium pasteurianum	205
Clostridium thermoaceticum	170
Clostridium innocuum	205

Example 2

Three of the species listed in Table 1, viz *Bacillus subtilis*, *Clostridium pasteurianum* and *Clostridium innocuum* gave PCR products of indistinguishable size. They were differentiated by their ability to hybridize with oligonucleotides corresponding to unique segments of the variable linker region of *spo0A*.

The DNA fragments were transferred by standard capillary blotting methods to a nylon membrane (Hybond N, Amersham) and hybridized under stringent conditions with 24-mer oligonucleotides (see below) that had been labelled at their 3' ends with digoxigenin, using a commercially available 3'-end labelling kit (Boehringer Mannheim). In each case, the oligonucleotide specifically detected the PCR product derived from the organism from which the primer had been synthesized; none of the PCR products derived from any of the other organisms were detected. The sequences of the oligonucleotides employed in this experiment, together with the sequences of the corresponding region of the spo0A linker region, were as indicated in Table 2.

Table 2

Organism	Peptide	Oligonucleotide
B. subtilis	NASSVTHR	AATGCCAGGAGTGTGACGCATCGT
C. pasteurianum	KAADVKIS	AAAGCTGCAGATGTAAAGATTTCT
C. innocuum	EHISSNVL	GAGCATATATCCAGCAATGTGCTT

Example 3

Amplification of variable length products across the intergenic region separating *spoIVB* and *spo0A*.

A pair of primers ATIGARTGGATGYT and TAICCYTTIATRTGIGCIGGIACICC, complementary to residues 407-411 (IEWML) of *spoIVB* from *B.subtilis* and 168-174 (PAHIKGY - reverse)

of *spoOA* from *B.subtilis* were used to amplify DNA segments fr m several bacilli and clostridia using a Perkin Elmer GeneAmp™PCR System 2400. Each reaction tube contained 100 pmole of each primer, 0.5μg of target DNA, all four dNTPs at 200μM each, 50mM KCl, 2mM $MgCl_2$, 10mM Tris/HCl, 0.01% gelatin (Sigma), pH 8.0, and 2.5 U of AmpliTaq DNA polymerase (Perkin Elmer Cetus). After denaturation for 5 min at 94°C samples were amplified for 35 cycles comprising a 40s denaturation period at 94°C, a 40s annealing period at 40°C and a 40s extension period at 72°C. Amplified products were analysed by electrophoresis through a 1.5% agarose gel. The results obtained are given in Table 3.

Table 3

Organism	Approx. Size of Amplified DNA Fragment (bp)
Bacillus subtilis	860
Bacillus megaterium	760
Clostridium beijerinckii	1200
Clostridium pasteurianum	820
Clostridium thermoaceticum	700

CLAIMS:

1. A method of monitoring endospore-forming bacteria present in a sample, which method comprises the steps of adding to said sample primers which are specific for conserved regions in a coding region in the genome of said bacteria, which coding region comprising two domains with a linker region connecting the two domains, and which coding region is characteristic of an endospore-forming bacterium, carrying out a DNA amplification procedure so as to amplify conserved regions of the coding region present in said sample and analysing for the conserved regions.

2. A method according to claim 1, wherein said coding region comprises an individual gene, said conserved regions comprising the carboxy and amino terminal domains of said gene.

3. A method according to claim 2 wherein said gene comprises *spoOA*.

4. A method according to claim 1, wherein said coding region comprises segments of at least two adjacent genes, each of said genes coding for a distinguishable product.

5. A method according to claim 4, where said adjacent genes comprise *spoIVB* and *spoOA*.

6. A method of identifying endospore-forming bacteria which comprises the steps of adding primers to a sample containing endospore-forming bacteria, said primers being specific for the conserved regions of a coding region in the genome of said bacteria which coding region has a two-domain structure and is characteristic of an endospore-forming bacterium, carrying out a DNA amplification step so as to amplify conserved regions of the coding region and

analysing the amplified DNA obtained, wherein said primers are such that they simultaneously amplify a DNA linker region of variable length connecting the conserved regions, the length of the DNA fragment obtained from said amplification step being characteristic of a particular endospore-forming bacterium.

7. A method according to claim 6, wherein said coding region comprises an individual gene, said conserved regions comprising the carboxy and amino terminal domains of said gene.

8. A method according to claim 7, wherein said gene comprises *spoOA*.

9. A method according to claim 6, wherein said coding region comprises segments of at least two separate adjacent genes coding for distinguishable products.

10 A method according to claim 9, wherein said adjacent genes comprise *spoIVB* and *spoOA*

11. A method according to any preceding claim wherein said amplification step comprises polymerase chain reaction amplification or isothermal amplification.

INTERNATIONAL SEARCH REPORT

International Application No
PC1/GB 95/02418

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 C12P19/34 C07H21/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	MOLECULAR MICROBIOLOGY, vol. 14, no. 3, November 1994 pages 411-26, BROWN, P. ET AL 'Characterization of spoOA homologues in diverse Bacillus and Clostridium species identifies a probable DNA-binding domain' entire document, especially "Conslusions," pages 442-423. ---	1-11
A	EP,A,0 502 271 (THE STANDARD OIL CO.) 9 September 1992 see claims 1-17 ---	1,6
A	EP,A,0 409 159 (SCHIMADZU CORP) 23 January 1991 see claims 11,30 --- -/--	1,6

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Name and mailing address of the ISA	Authorized officer
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF BACTERIOLOGY, vol. 172, no. 3, March 1990 pages 1306-11, VAN HOY, B. ET AL 'Characterization of the spoIVB and recN loci of Bacillus subtilis'	

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INTERNATIONAL SEARCH REPORT

...information on patent family members

Intern...nal Application No
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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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		JP-B-	7089958	04-10-95
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